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Alterations in genetic pathways following radiotherapy for head and neck cancer

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Abstract

Background: Radiotherapy (RT) is an integral component in the treatment of head and neck cancer (HNC). We hypothesized there would be alterations in gene-expression and pathway activity in HNC samples obtained in recurrent HNC that were previously treated with RT, when compared to RT-naïve disease.

Methods: Patient data was abstracted from a prospectively maintained database. Linear-microarray analysis and supervised gene-set enrichment-analysis were employed to compare RT-naïve and recurrent disease after prior-RT.

Results: A total of 157 patients were analyzed, 96 (61%) were RT-naïve and 61 (39%) had RT. After radiation, there was upregulation of genes associated with angiogenesis, protein-translation-machinery, cell-cycle regulation, and growth factors, and downregulation associated with Myc activity, and hypoxic response (all $P < .001$). Previously irradiated HNC was associated with down-regulation in 19/42 genes in the Wnt/B-catenin-pathway ($P = .045$) and 119/199 genes involved in the MYC target pathway ($P = .024$).

Conclusion: Patients with recurrences salvaged surgically post-RT had significant alterations in gene-expression and in Wnt/B-catenin and MYC-target pathways. These pathways may represent potential targets to prevent development of resistance to RT.

Keywords

genetic pathways; head and neck cancer; radioresistance; radiotherapy

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interests.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

1 | INTRODUCTION

Radiotherapy (RT) is an integral component in the curative setting for head and neck cancer (HNC), both as a definitive modality and adjuvant to surgery.¹ For locally or regionally advanced HNC, randomized trials have shown improvements in control with either accelerated RT or concurrent chemotherapy.^{2–5} Despite this intense therapy, 10–40% of patients nevertheless recur.^{2–5} The mechanisms of radioresistance in HNC are poorly understood. In vitro, gene expression can be altered in response to RT in HNC cell lines.⁶ Relapse after definitive RT may be due to innate and/or acquired radioresistance of the surviving colonies.⁷

To better understand radiation's effect on HNC pathways in vivo, we investigated whether HNC that recurred after definitive RT would have an altered gene expression compared to HNC samples that were RT naïve. The purpose of our study is to identify the genes and pathways involved in HNC response to RT and radioresistance, which could serve as novel biomarkers or therapeutic targets.

2 | METHODS

2.1 | Patient methods

After IRB approval, the Total Cancer Care (TCC) database was queried for squamous cell carcinomas of the head and neck from primary mucosal sites (eg, oropharynx, larynx, oral cavity, or hypopharynx).⁸ Patient data was abstracted from a prospectively maintained institutional database and chart review. A total of 238 cases were identified. After excluding non-squamous histologies, distant metastases, and insufficient data, 157 patients were left for analysis. Specimens were obtained from salvage surgery following prior radiotherapy in 61 patients (“post-RT”), or from 96 patients without prior radiotherapy (“RT naïve”).

2.2 | Statistical methods

Patient and tumor characteristics between RT naïve and post-RT samples were compared via Pearson's chi-square or Fisher's exact test for categorical variables, and Wilcoxon rank sum for continuous variables, as appropriate. All 157 Rosetta/Merck Human RSTA Custom Affymetrix 2.0 microarrays were normalized using iterative rank-order normalization and experimental batch effects were examined using a principal component-based cluster analysis.⁹ Linear models for microarray analysis (LIMMA) was employed to identify individual genes with differential expression between groups of samples obtained before or after RT.¹⁰ A biological knowledge-based Gene Set Enrichment Analysis (GSEA) was further performed to determine whether genes in 50 well-annotated hallmark gene pathways show significantly concordant differential expressions between the two groups.¹¹ Statistical significance is defined when false discovery rate (FDR)-adjusted *P*-value <.05 for a multiple testing correction in the individual gene analysis and nominal *P*-value <.05 for GSEA in an exploratory manner.¹²

3 | RESULTS

3.1 | Patient characteristics

A total of 157 patients were analyzed. A total of 96 (61%) patients were RT naïve and 61 (39%) post-RT. Median follow up for all patients was 19 months. Patient characteristics are shown in Table 1. Patients were well balanced, except primary site ($P = .004$) and tumor classification between RT naïve vs post-RT (T3/T4: 47% vs 25%, $P = .007$). A total of 27 patients received concurrent chemotherapy during RT, most commonly cisplatin alone with other regimens (in decreasing frequency) including cisplatin with cetuximab, cetuximab alone, carboplatin/paclitaxel, or single agent carboplatin.

3.2 | Gene expression

At an FDR of <0.05 , there were 251 genes that were differentially expressed in the two cohorts (Supplemental Table S1). After radiation, the most notable differences were upregulation of genes associated with angiogenesis (*ENPP5*), protein translation machinery (*RPL23AP53*), cell cycle regulation (*CMTM4*), radiosensitivity (*GDF15*), development (*HOXB6*), and growth factors (*VEGFB*, *IGFBPL1*), and downregulation associated with mitochondrial dysfunction (*DDHD1*), ubiquitination (*PSMD6*), Myc activity (*MYCBP2*, *MAX*), and hypoxic response (*HIF1AN*) (all LIMMA $P < .001$) (Table 2).

3.3 | Pathway analysis

Two pathways were significantly altered, including the Wnt/B-catenin pathway and MYC-target pathway. Previously irradiated HNC was associated with a downregulation in 19 of 42 genes in the Wnt/B-catenin pathway (GSEA $P = .045$) and 119 of 199 genes involved in the MYC target pathway (GSEA $P = .024$) (Figure 1).

In the Wnt pathway, there was a significant difference in the expression of 14 genes (Table 3). In previously irradiated HNC, there was a down-regulation of nine Wnt pathway genes, many of which have a recognized association with MYC activation (*CCND2*, *PPARD*, *SKP2*, *TCF7*, *MAML1*, *AXIN2*, *AXIN1*), and up-regulation of five genes, majority of which are associated with histone de-acetylation/acetylation (*NCOR2*, *HDAC5*, *KAT2A*, *HDAC11*) (all LIMMA $P < .05$). In the Wnt pathway, the most dramatic difference in gene expression is a downregulation in *CCND2* in previously irradiated HNC.

In the MYC pathway, there were 44 genes that were differentially expressed in the two cohorts, with down-regulation of 42 genes in the previously irradiated (Table 4). Many of the genes downregulated were associated with RNA splicing/maturation ($n = 12$), protein translation ($n = 10$), and proteasome/ubiquitination process ($n = 8$). The largest difference in the MYC pathway was a 1.35-fold decrease in *SRPK1* expression in previously irradiated HNC.

4 | DISCUSSION

To our knowledge, this is one of the first in vivo studies comparing the differences in gene and pathway expression in RT naïve HNC vs recurrent previously irradiated HNC samples. Our study identified a differential expression of various genes, with previously described

associations with HNC stem cells,¹³ proliferation,^{14–16} and radioresistance.^{14,17–19} Most notably, our post-RT samples had a significant downregulation of the Wnt and Myc pathways.

4.1 | Notable gene expression

Post-RT samples had a significant change in the expression of 251 genes, some of which have been associated with tumor suppression,^{20,21} radiosensitivity,^{14,18,19} hypoxic induction,²² and angiogenesis.²³

4.2 | Tumor suppressors

Loss of tumor suppressor function can lead to aberrant growth by disinhibition of the cell-cycle machinery, which has been described as a mechanism for HNSCC carcinogenesis.²⁴ In this study, previously irradiated samples had a significant upregulation of tumor suppressor genes insulin growth factor binding protein-like 1 (*IGFBPL1* or *IGFBP-rP4*), and chemokine-like factor (CKLF)-like MARVEL transmembrane domain containing four (*CMTM4*). Insulin growth factors can act as a tumor suppressor by p21 induction independent of the p53 pathway.²¹ IGFBP-rP4 can affect the interaction between growth factors and cell surface receptors, and has been suggested as a novel putative tumor suppressor in vitro.²⁰ *CMTM4* is a tumor suppressor gene characterized by G2/M phase regulation, p21 upregulation, and inhibition of cell migration.^{15,16} The downregulation of *CMTM4* has been described in clear cell renal cell carcinoma, with restoration of expression significantly suppressing cell growth in vitro and in xenograft models.¹⁵ Our study suggests that the decreased expression of these tumor suppressor genes may play a larger role in the carcinogenesis and progression of RT naive tumors, but may have a limited role in the growth of recurrent previously irradiated HNC.

4.3 | Radiosensitivity

Since RT is integral to the treatment of HNC, understanding the radiosensitivity of this disease may help prognostically stratify HNC patients treated with RT and provide potential genetic targets that can improve RT's efficacy, which will eventually lead to a personalized treatment approach in HNC.²⁵ Several genes identified in our study have been associated with radioresistance, including: Cyclin D2 (*CCND2*),¹⁷ vascular endothelial growth factor B (*VEGFB*),¹⁸ and growth differentiation factor 15 (*GDF15*).^{14,19}

Methylation and decreased expression of Cyclin D2 has been associated with radioresistance in HNSCC.¹⁷ In our study, post-RT was also associated with a decreased expression of Cyclin D2, which supports the claim that these recurrent HNCs have a differential gene expression more consistent with radioresistant HNSCC. In HNSCC, VEGFB is a ligand associated with cell survival and radioresistance, through activation of FLT1 receptor tyrosine kinase (VEGFR1).¹⁸ There is no evidence that VEGFB's contribution to radioresistance is inducible, whereas GDF-15 may be induced by radiation.¹⁹ GDF-15 is a cytokine in the TGF-B family, and has been associated with carcinogenesis and tumor progression of oral squamous cell carcinoma.¹⁴ GDF-15 expression is associated with radioresistance through anti-apoptotic effects.¹⁴ The upregulation of GDF-15 in response to radiation is consistent with our study, which showed continued elevation in recurrent post-

RT HNC. Our study suggests that the radiation induced upregulation of GDF-15 may be a late responding and persistent effect that may contribute to the tumor growth and radioresistance in recurrent HNC. Our study also suggests that VEGF and GDF-15 may predict for innate and inducible biomarkers of radioresistant HNC, and identifies novel therapeutic targets that may improve HNC radiosensitivity.

4.4 | Hypoxia and angiogenesis

Tumor hypoxia can lead to detriment in outcome because it can lead to a decreased RT efficacy, increased tumor invasiveness, loss of apoptosis, and poorly structured angiogenesis that further exacerbates the hypoxic state.²⁶ In our study, post-RT HNC had a difference in the expression of genes associated with hypoxia and angiogenesis, consisting of a downregulation in factor-inhibiting hypoxia-inducible-factor 1 (*FIH-1*)²² and upregulation of ectonucleotide pyrophosphatase/phosphodiesterase 5 (*ENPP5*),²³ which have also been seen in glioblastoma (GBM). Hypoxia-inducible-factor 1 (*HIF-1*) regulates the transcription of growth factors and cytokines in response to hypoxia, and can cause tumor progression, tumor growth, and vascularization.²² The increased expression of *HIF-1* has been associated with poor prognosis in several HNCs including the oral cavity^{27,28} and nasopharynx.²⁹ Factor-Inhibiting HIF-1 (*FIH-1* or *HIF1AN*), a potent regulator of HIF-1, is often deleted in GBM.²² The loss of FIH-1 can lead to constitutively active HIF-1, which may contribute to GBM survival during hypoxia, hypervascularization, and tumorigenesis.²² A putative angiogenesis associated gene, *ENPP5*, was also upregulated in GBM.

Since GBM is historically considered radioresistant,³⁰ the similarity in expression of hypoxic/angiogenesis related genes in GBM with our post-RT cohort, suggests these may be involved in radioresistance. This may also provide novel targets and biomarkers that can improve HNC treated with radiation.

4.5 | Pathway analysis

The Wnt pathway has many functions including differentiation, migration, and proliferation.³¹ It is involved in the activation of several downstream factors including cyclin D and myc, and has been associated with HNC transformation, such as oral carcinogenesis.^{32,33} Tumor stress such as hypoxia³⁴ and radiation³⁵ have been associated with decreased Wnt pathway signaling. This is consistent with the findings in our study, where previously irradiated HNC had a significant decrease in the Wnt pathway.

Myc, a known target of the Wnt pathway, contributes to cell proliferation, cell growth, and tumorigenesis.³⁶ Previous studies have shown that radiation can decrease the expression of Wnt targets, including Myc.³⁵ In our post-RT samples, majority of the downregulated genes in the Wnt pathway were involved in Myc pathway activation, the most notable of which is Cyclin D2 (*CCND2*). Cyclin D2 expression, a gene primarily responsible for G1/S cell cycle progression, has been associated with HNSCC carcinogenesis³⁷ and invasiveness,³⁸ whereas decreased expression of *CCND2* has been associated with radioresistance.¹⁷ We hypothesize that the Cyclin D2 expression and the Wnt/Myc pathway may be the initial driving mechanism for the carcinogenesis of non-irradiated HNC, but after exposure to radiation, a downregulation of these pathways increase radioresistance and improving cell survival.

4.6 | Strengths and limitations

This is one of the first studies to analyze differences in HNSCC gene expression pathways following RT in vivo. Some of the strengths of our study include: a relatively large study, ability to capture inducible factors for altered gene and pathway expression, and in vivo samples allowing a closer clinical correlation than cell lines. This study evaluates the genetics of all HNSCC, assuming that all HNSCC share common carcinogenesis pathway. Although HNSCC carcinogenesis is historically related to smoking, there is a subset driven by human papilloma virus (HPV) infection, which was not available in our tumor samples. Our cohort is primarily made up of current and former smokers (66%), 75% of which have a > 10 pack-year smoking history, suggesting that the majority of these patients likely experienced carcinogenesis related to tobacco exposure. When comparing RT naïve vs post-RT, there was a difference in the primary site of disease, but no difference in smoking status ($P = .402$) or pack-year history ($P = .259$). This suggests that differences in pathway expression are less likely due to initial tumorigenesis or influence by HPV infection, and more likely associated with prior RT. A proportion of patients receiving RT also received concurrent chemotherapy, and the impact of radiosensitizing chemotherapy on the HNSCC gene expression pathways is not entirely clear and could limit the interpretation of these results. Future prospective studies, with a larger cohort and HPV data, are required to further elucidate the pathways responsible for radioresistance in HNC. The ideal method of correlating RT's effect on HNC would be to analyze matched RT naïve and post-RT samples, which will be implemented in future prospective protocol design. Validation of radioresistant genes and pathways requires testing in an external dataset, along with in vitro radiosensitivity studies with gene and pathway manipulation. The addition of RT naïve and post-RT normal tissue could also be implemented in future protocol design.

5 | CONCLUSION

Patients with recurrences salvaged surgically after prior RT had significant alterations in gene expression and in the Wnt/B-catenin and MYC target pathways. There were also changes in gene expression after RT that is similar to changes seen in radioresistant tumors. This suggests that the pathways involved in HNC carcinogenesis and initial progression differs from the mechanism responsible for inherent or acquired radioresistance in HNC. These differentially expressed genes may serve as potential biomarkers or as novel therapeutic targets to help select treatments or improve outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Heat map correlating RT exposure and gene expression. Increased (red) or decreased (blue) gene expression is correlated with RT naïve and post-RT cohorts. There were 199 canonical genes in the Myc target pathway (A) and 42 genes in the Wnt-beta catenin pathway (B)

TABLE 1

Patient and tumor characteristics

	Total (n = 157)			RT naïve (n = 96)		Post-RT (n = 61)		P value (Chi-square; * Fisher's exact)
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	
Race								.416
White	142 (90%)	86 (90%)	56 (92%)					
Black	6 (4%)	5 (5%)	1 (2%)					
Other	1 (1%)	0 (0%)	1 (2%)					
Unknown	8 (5%)	5 (5%)	3 (5%)					
Gender								.472*
Female	44 (28%)	29 (30%)	15 (25%)					
Male	113 (72%)	67 (70%)	46 (75%)					
Grade								.369
Well	21 (13%)	14 (15%)	7 (12%)					
Moderate	76 (48%)	49 (51%)	27 (44%)					
Poor	36 (23%)	22 (23%)	14 (23%)					
Undifferentiated	1 (1%)	1 (1%)	0 (0%)					
Unknown	23 (15%)	10 (10%)	13 (21%)					
Primary site								.004
Oropharynx	28 (18%)	9 (9%)	19 (31%)					
Oral cavity	72 (46%)	53 (55%)	19 (31%)					
Larynx	36 (23%)	18 (19%)	18 (30%)					
Hypopharynx	7 (5%)	6 (6%)	1 (2%)					
Nasal cavity/sinus	8 (5%)	6 (6%)	2 (3%)					
Other	4 (3%)	3 (3%)	1 (2%)					
Unknown primary	2 (1%)	1 (1%)	1 (2%)					
Tumor classification								.007*
TX-T2	97 (62%)	51 (53%)	46 (75%)					
T3/T4	60 (38%)	45 (47%)	15 (25%)					
Nodal classification								.945
N0/NX	102 (65%)	62 (65%)	40 (66%)					
N1	19 (12%)	11 (12%)	8 (13%)					
N2	34 (22%)	22 (23%)	12 (20%)					
N3	2 (1%)	1 (1%)	1 (2%)					

	Total (n = 157)	RT naïve (n = 96)	Post-RT (n = 61)	P value
	No. (%)	No. (%)	No. (%)	(Chi-square; * Fisher's exact)
M classification				
M0/MX	156 (99%)	95 (99%)	61 (100%)	>.999
M1	1 (1%)	1 (1%)	0 (0%)	
Smoking status				.402
Never	22 (14%)	17 (18%)	5 (8%)	
Current	47 (30%)	27 (28%)	20 (33%)	
Former	57 (36%)	33 (34%)	24 (39%)	
Unknown	31 (20%)	19 (20%)	12 (20%)	
Pack-years				.259
<=10	33 (21%)	25 (26%)	8 (13%)	
10 to 20	10 (6%)	5 (5%)	5 (8%)	
>20	75 (48%)	43 (45%)	32 (53%)	
Unknown	39 (25%)	23 (24%)	16 (26%)	
	Median (range)	Median (range)	Median (range)	P value (Mann-U-Whitney)
Age	63 (23-94)	63 (26-94)	60 (23-85)	.461
Smoking	30 (0-150)	30 (0-150)	36 (0-90)	.361

Abbreviation: RT, radiotherapy.

* P value based on Fisher's Exact test.

TABLE 2

Gene expression changes associated with radiation exposure

Function	Gene	RT naïve (mean)	Post-RT (mean)	Fold change	P value	FDR Q value
Angiogenesis	<i>ENPP5</i>	28.5	71.9	0.4	<.001	.003
Protein translation machinery	<i>RPL23AP53</i>	67.6	102.3	0.66	<.001	.008
Cell cycle regulation	<i>CMTM4</i>	377.2	513.7	0.73	<.001	.008
Radiosensitivity	<i>GDF15</i>	122.5	200.2	0.61	<.001	.008
Development	<i>HOXB6</i>	30.2	49.9	0.61	<.001	.008
Growth factors	<i>VEGFB</i>	1503.7	1913.6	0.79	<.001	.024
	<i>IGFBPL1</i>	77.4	279	0.28	<.001	.031
Mitochondrial dysfunction	<i>DDHD1</i>	53.7	33.7	1.6	<.001	.008
Ubiquitination	<i>PSMD6</i>	1460.4	1228.5	1.19	<.001	.008
Myc activity	<i>MYCBP2</i>	859.3	672.2	1.28	<.001	.044
	<i>MAX</i>	95.8	70.3	1.36	<.001	.025
Hypoxic response	<i>HIF1AN</i>	132.9	96	1.38	<.001	.015

TABLE 3

Radiation induced changes in Wnt pathway

Function	Gene	Gene enrichment score	Fold Change (RT naïve/post-RT)	DE LIMMA (P value)	DE LIMMA FDR (Q value)
Myc activation	<i>CCND2</i>	0.071	1.61	.003	.095
	<i>PEP2</i>	0.174	1.36	.009	.149
	<i>SKP2</i>	0.228	1.34	.007	.14
	<i>TCF7</i>	0.278	1.23	.003	.098
	<i>MAML1</i>	0.334	1.12	.017	.192
Histone de-acetylation/acetylation	<i>AXIN2</i>	0.253	1.1	.011	.161
	<i>AXIN1</i>	-0.022	1.09	.017	.195
	<i>NCOR2</i>	0.002	0.94	.003	.095
	<i>HDAC5</i>	-0.009	0.89	.013	.175
	<i>KAT2A</i>	0.029	0.85	.009	.153
	<i>HDAC11</i>	-0.024	0.79	.001	.074

TABLE 4

Radiation induced changes in Myc pathway

Function	Gene	Gene enrichment score	Fold Change (RT naïve/post-RT)	DE LIMMA (P value)	DE LIMMA FDR (q value)
RNA splicing/maturation	<i>SRPK1</i>	0.051	1.35	<.001	.043
	<i>SNRBD1</i>	0.132	1.22	.002	.084
	<i>DDX18</i>	0.349	1.22	.008	.141
	<i>SRSF1</i>	0.417	1.18	.003	.099
	<i>C1QBP</i>	0.225	1.18	.002	.081
	<i>NCBP1</i>	0.317	1.17	.001	.071
	<i>DDX21</i>	0.196	1.14	.007	.14
	<i>HNRNPR</i>	0.211	1.13	.002	.089
	<i>EXOSC7</i>	0.371	1.12	.01	.157
	<i>SERBP1</i>	0.349	1.1	.002	.091
	<i>DHX15</i>	0.426	1.1	.002	.081
	<i>SRSF3</i>	0.355	1.09	.001	.069
Protein translation	<i>EIF4E</i>	0.322	1.35	<.001	.017
	<i>RPS6</i>	0.079	1.26	.017	.192
	<i>PA2G4</i>	0.273	1.18	.006	.13
	<i>SRSF1</i>	0.417	1.18	.003	.099
	<i>EIF2S1</i>	0.253	1.15	.003	.101
	<i>ETF1</i>	0.379	1.13	.007	.133
	<i>RPL22</i>	0.37	1.12	.015	.182
	<i>EIF4H</i>	0.4	1.11	.001	.076
	<i>EIF4A1</i>	0.433	1.11	.002	.087
	<i>RPL14</i>	0.335	1.05	.007	.141
	<i>UBE2E1</i>	0.028	1.21	.007	.139
Proteasome/ubiquitination process	<i>PSMD1</i>	0.269	1.23	.001	.059
	<i>PSMB2</i>	0.419	1.18	.008	.147
	<i>PSMC6</i>	0.298	1.16	.001	.071

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Function	Gene	Gene enrichment score	Fold Change (RT naïve/post-RT)	DE LIMMA (P value)	DE LIMMA FDR (q value)
	<i>COPS5</i>	0.2	1.16	.002	.082
	<i>PSMA2</i>	0.107	1.14	<.001	.034
	<i>UBE2L3</i>	0.346	1.13	.003	.095
	<i>PSMD14</i>	0.408	1.11	.007	.134